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QUANTITATIVE ELEMENTAL MAPPING OF BIOMEDICAL SPECIMENS USING THE NUCLEAR MICROPROBE.

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Abstract

Quantitative and structural elemental information is available from the nuclear microprobe through a time consuming fit of the (X-ray) spectrum at each point. An alternative technique is proposed which greatly shortens the analysis procedure and allows an increased number of samples to be processed. The method discussed here is to create elemental maps which, when they are divided by the charge/pixel and multiplied by a scaling factor, will form quantitative maps. The scaling factors are obtained from a calibration procedure comparing a large number of fitted X-ray spectra with the corresponding contents of selected energy windows. The technique also allows the reduction of artefacts due to spectral overlap, assuming that a simple background model can be used.

Key Words: Micro analysis, elemental mapping, PIXE, back-scattering, nuclear microprobe, quantification, rat, retina, blue light

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Introduction

The scanning nuclear microprobe (NMP) is a powerful instrument applied in various fields (Lindh, proceedings 1993). In biomedical research the NMP interest is focused on establishing knowledge about elemental distributions of interest for physiology and metabolic activity under normal and abnormal conditions. The biological relevance of the achieved results must always be taken into account; how well does the data from the sample describe the situation in the living matter, how typical is the situation in the living matter relative to the overall aim of the survey? More precisely, as there is a biological variability on all scales, from individuals, through organs and cell structures down to the single cell, and as this variability is not precisely known, one must try to compensate for it by analysing a sufficient number of samples. Analysing many samples for quantitative results is a very time consuming procedure both in accelerator beam time and in data evaluation time. This paper discusses a way to shorten the data evaluation time and at the same time achieve quantitative elemental maps of the analysed samples thus increasing the possibilities of understanding biological behaviour.

The method proposed here is to create elemental maps which, when they are divided by the charge/pixel and multiplied by a scaling factor, will form quantitative maps. The scaling factors are obtained from a calibration procedure comparing fitted X-ray spectra with the corresponding contents of selected energy windows. The X-ray fitting provides necessary information on detector efficiency, transmission factors, cross sections, etc., which for each element is reduced into the scaling factor, converting the counts/unit charge to areal mass density.

Material and Methods

The study reported here is part of a larger pilot survey covering analyses of 12 rat retina samples with the aim of studying changes in ion-concentration as a function of strong blue light irradiation (Chen 1993). For evaluation of the method proposed, four similar retina sections were examined for their contents of Cl, K, Ca and Fe using both a standard spectral fitting technique and an elemental windowing technique.

Sample preparation.

The animals were exposed to different doses of blue light. One hour after the end of exposure, the exposed eyes were removed immediately after sacrifice and were quenched in isopentane precooled to -140 °C by liquid nitrogen. The frozen eyeball was then cryosectioned in the horizontal plane to a section of 20 µm. The cryosections were freeze-dried and a section near the optical nerve was selected from each eye for nuclear microprobe analysis

Instrumentation and analytical methods.

The analyses were performed at the new Lund Nuclear Microprobe (Malmqvist et al., 1993), using 2.4 MeV protons, a beam size of 8*8 μm^2, and a beam current of 400 pA. The dwell time per pixel was 10 ms per scan, the sample was scanned repeatedly until satisfactory statistics was achieved; the total time for analysis per sample was 1 - 2 hours. The analytical techniques used were PIXE (Particle Induced X-ray Emission) and detection of (elastically) backscattered protons. The X-rays were detected using a Si(Li)-detector in 135 degrees and the backscattered protons with a silicon surface barrier detector in 140 degrees. All data from the scanning beam was collected and stored in event-by-event mode using a VME-bus system and a Micro VAX II (Lövestam 1989). From the stored data, both background corrected elemental maps and series of spectra were obtained using modified versions of the code discussed by Pallon (1990), now running on a DEC 3000 Open VMS AXP workstation (64 bit architecture).

Production of elemental images.

The images shown in this paper were produced by doing screen dumps into local files on a PC that was emulating a Tektronix 4107 terminal (EMU-TEK™, FTG-Data Systems). The files were converted to *.bmp (bit mapped) files, imported to a standard graphics/image program (here HiJaak) running under Windows, grey-level adjusted and printed out on a 600 dpi Laser Printer.

Target mass density measurement.

The method used to measure the target's mass density is a modification of the mass normalisation procedure using elastically backscattered protons suggested by Themner and Malmqvist (1986). For calibration, a special sample consisting of two Kapton films of different areal density overlapped to give three areal densities was analysed with the scanning beam. Using the known areal mass densities of this sample, calibration of the number of elastically backscattered protons in a selected energy interval per unit charge for unit mass density was obtained and the values used to create elemental maps of the retinal sample mass density. A secondary control for each analysed sample was to compare the calculated areal mass density of the backing foil displayed in the elemental map against the known value.

It was investigated whether the secondary electron induced background in the PIXE spectrum could be used as a sample mass density parameter in the same way as elastically backscattered protons for the case that only PIXE analysis had been performed. One approach of this method is to use "an interference free region of the background with no characteristic peaks". This is unrealistic for single point spectra from NMP analysis, as too few counts would be present in a useful region. As the characteristic peaks could be large compared to the background, the use of a computer fitted background from the spectral fitting code (HEX), (Johansson, 1982) was necessary.

Data evaluation.

The fast method of analyzing PIXE data is to record only the total number of counts in certain predefined energy windows selected for the elements of interest in the sample. The most exact method - seldom applied to elemental maps - is to fit the spectrum of each analysed point to yield elemental contents and concentrations. Various methods of spectral fitting exist, see e.g. Van Espen and Janssens, (1993) and Campbell et al. (1986). As the main method of elemental analysis in NMP work is PIXE, a large number of X-ray spectra must be evaluated (4096 for a 64*64 pixels scan) using some suitable, reliable and stable code. The typical fitting time of a PIXE spectrum is about one minute on a Digital MicroVAX II computer (from 1986) although with the computers available today, the time is reduced to seconds. Assuming that the charge at each point is known, quantification will give the areal mass density of the elements (e.g. in ng/cm^2). To calculate the concentration of the elements of the sample in units of ppm (e.g. ng/mg), the local areal mass density must be known from, for example, the number of backscattered ions or the energy loss of the transmitted ions using Scanning Transmission Ion Microscopy, STIM, (on-axis or off-axis). By dividing the elemental content of each pixel by its corresponding mass density, elemental maps showing the concentrations of the elements can be achieved. However, the time necessary for X-ray fitting of each pixel is long and is thus in strong conflict with the need to analyse many samples. Different approaches to this problems are found in the literature. Grime (1993) discusses a technique to quickly obtain quantitative line-scans across a sample by using energy windows. The counts in each window are converted to concentrations by using factors obtained from a fit of the total scan spectrum. In a recent paper, Ryan and Jamieson (1993) introduces a scheme for spectral decomposition in live time, called dynamic analysis, based on the deconvolution of a master spectrum. From matrix transformations, accurate elemental mapping and resolving of spectral interferences is provided in live time, which was demonstrated on geological samples.

In this work, another compromise between using pure elemental maps from energy windows and exact fitting of each spectrum is suggested. The procedure takes place in four steps:

I) **Elemental maps:** X-ray data is sorted into background subtracted elemental maps having energy windows with limits taken from an X-ray fit of the full scan spectrum which has good statistics. The data sorted into a given window thus represents the peak area of that X-ray line after background subtraction. The choice of method or procedure for background subtraction may be critical to the time and accuracy of the analysis. A technique is described by Lövestam and Swietlicki (1992) where the background is removed by a combination of Fourier transformation, low frequency filtering and inverse transformation. However, in this case all the data must be available in spectral form. In the present work, the background is estimated as the mean value of the contents of the channels limiting the energy window, a technique that works well for well defined lines of a spectrum, but will be influenced by possible spectral overlap.

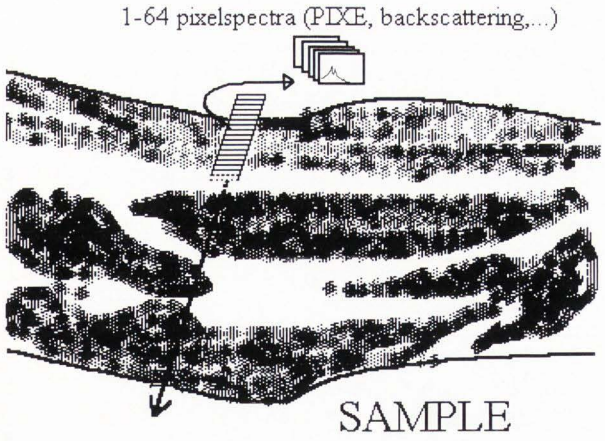


Fig. 1. Principal figure showing how single point spectra were selected in a line across the sample from the complete data set. Data from a small rectangle (here 1*6 pixels) is merged into one data point.

II) **Calibration points:** Series of points are selected from the overall dataset to represent both high and low concentration values. In this case, points in a line across the samples were used, see Fig. 1. Each series (or scan) consisted of about 40-55 single spectra, and three scans were selected on each sample. Thus a data basis of 130 - 160 points was obtained from each evaluated sample, and for each point there was both an X-ray spectrum and a backscattering spectrum. Using the X-ray spectra, both a complete spectrum fit was done yielding the elemental contents, and in parallel, the contents of the corresponding energy windows were obtained.

III) **Scaling factors:** Elemental contents as obtained both from spectral fitting and energy windows were then compared. By multiplying data from the energy window with a scaling factor, the two data sets were matched for maximum overlap. This scaling factor was found to be rather constant within the same sample, see Table 1.

IV) **Quantitative maps:** By multiplying the elemental maps with the average scaling factors previously obtained, quantitative maps were achieved.

This procedure was checked for the elements Cl and K in the four different samples. It was also applied to the minority element Fe and the interfering element Ca in one of the samples. For the case of Ca, which is obtained from a window around the K_α peak at 3.691 keV, there is an interference from K K_β line at 3.589 keV. The ratio between the K K_α and the K_β line is 0.121 for thin, organic matrices, thus a corresponding part of the K-window content was subtracted from the Ca-window.

Results and Discussion

Quantification

The results of the comparison of the two evaluation methods are shown for a number of scans in Figs. 2 and 3. The figures are selected to be typical for the results. The variation in the scaling factor determined at each scan is small and shown in Table 1. As can be seen, within the same sample, the factor is constant for K and varies little for Cl. Between the samples, the factor varies for Cl and is rather

Table 1. Scaling factors [(ng/cm²)/(counts/nC] to transform the energy window contents into elemental content in ng/cm². Data from four different samples having two or three scans each.

		Scaling factor	
Sample number	Scan number	Chlorine	Potassium
1	1	37.5	17.5
1	2	35.0	17.5
2	1	31.0	15.5
2	2	30.0	15.5
2	3	30.0	15.5
3	1	27.0	15.0
3	2	27.0	15.0
3	3	27.0	15.0
4	1	28.5	15.0
4	2	30.0	15.0
4	3	30.0	15.0

constant for K. The agreement between the spectral fitting and energy windowing method is fairly good, provided that the scaling factors are adjusted for each sample, i.e. an average over each sample. For the case of Fe, being a minor element, but far away from interfering large peaks, the similar comparison is shown in Fig. 4, with good agreement whenever the contents are above the detection limits, which are around 300 ng/cm². For the case of Ca, which is interfered by K, results are shown in Fig. 5. The figure shows the contents of the uncorrected calcium peak and compares the fitted contents with the content determined from the energy window procedure corrected for the potassium K_β interference; the fitted potassium content is also displayed.

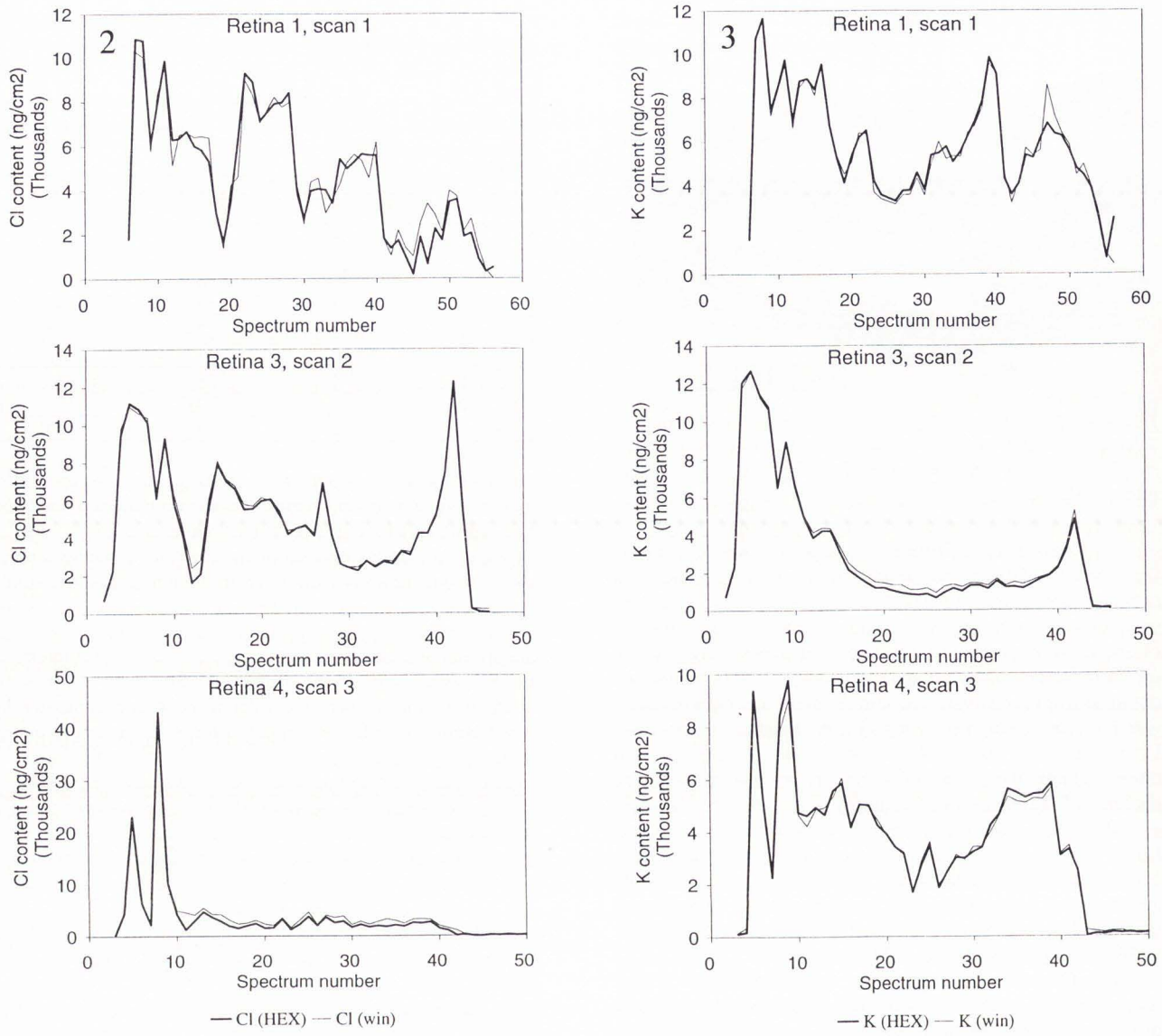
Two things can be noticed; that the uncorrected Ca-window is greatly influenced by the K content, and that the Ca-window after corrections quite well follows the fitted Ca-values. Thus it seems possible to decrease the K influence on the Ca elemental maps, see figure 6.

The good agreement for Cl and K is what could be expected as they represent cases where the X-ray peaks within the energy windows are large compared to the background. The difference between spectral fitting (Gaussian peak + background) and pure background subtracted energy windows should be minimal. For the case of small peaks however, the actual background level is more critical, and larger differences are expected.

Larger deviations should also be expected when an energy window is put around a minor peak having large neighbouring peaks. Their tails will then influence the background. The physics of tails is complex and not discussed further in detail; the important part here is that small peaks may be incorrectly estimated using the rather simple evaluation model proposed in this paper. For that reason it is important to select point spectra for calibration of the scaling factors from as many regions as possible in each sample. If interferences or overlaps are expected to be a serious problem, one could consider a more general approach, see e.g. Ryan and Jamieson (1993).

Mass density determination.

From the Kapton sample, the calibration factor between known areal mass densities and the number of backscattered protons was obtained and applied to all samples. When inspecting the elemental map of Retina 1, Fig. 7, one can



Figures 2 and 3. Comparison of Cl (Fig. 2, at left) and K (Fig. 3, at right) contents in points across the sample using both exact fitting (Cl-HEX) and energy window (Cl-win) techniques. See text for more details.

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notice that a region in the sample backing looks thinner than the rest. When studying the sample, it was found that one of the backing foils was partly broken (the sample is placed between two backing foils like a sandwich). In Fig. 7 the damaged part can be clearly seen as a dark band going on the diagonal having a mass density change of about 200 $\mu g/cm^2$ which corresponds to one layer of backing foil. This example illustrates that the use of backscattered protons for mass density normalisation is sensitive and safe.

Results of the areal mass density from the fitted X-ray spectra are shown in Fig. 8. The background used is calculated, based on a physical model, and fitted to each spectrum. For the comparison, a major part of the fitted secondary electron Bremsstrahlung background was used. The agreement is not satisfying, although the tendencies are parallel. One possible explanation for the poor agreement can be the limited number of counts, many times below 70, in the background of the pixel spectra. Having too few counts, the computer fitting becomes unstable, yielding too large variations in the background fit. There is a lower limit of total counts in the spectrum below which it is not possible to use the background fit. To find this lower limit, data from the Kapton sample of 1.36 mg/cm^2 was used forming X-ray spectra having background counts ranging between 20 and 2000. The corresponding backscattering spectra formed contained 10 to 20 times more counts due to higher detection efficiency. A comparison of the calculated mass densities using the fitted background and the number of backscattered protons as a function of the number of counts is shown in Fig. 9; values are expressed as the relative deviation from the known thickness. Clearly, for cases having less than 1000 counts, the mass density calculation using background fitting

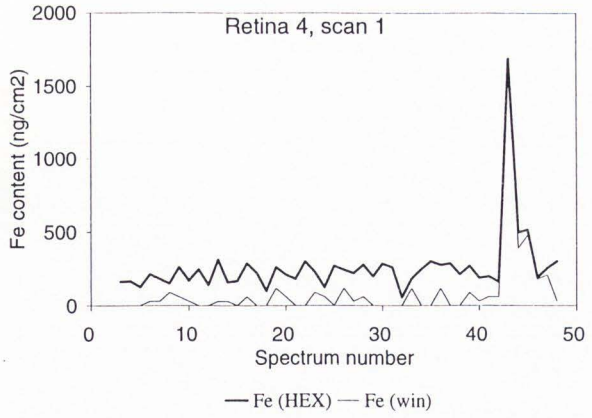

Fig. 4. As Fig. 2 but for Fe

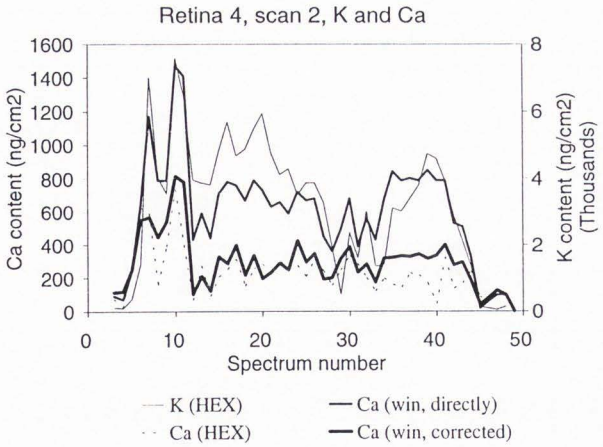

Fig. 5. As Fig. 2, but for Ca. The plot shows a) the interfering K-profile, b) the Ca energy window without correction, c) the spectral fit of Ca, and d) the energy window with an interfering part of the K energy window subtracted. Note the similarity of the uncorrected Ca-profile with the K profile showing its strong influence.

is rather uncertain. This is not the situation when using the backscattering method where the plot shows that the deviation is less than 5 % for most of the cases.

Conclusion

The fitted background as an areal mass density parameter is unreliable for NMP point spectra which often have poor statistics. It is also in principle in contradiction to the rest of this work which aims at minimising the use of spectral fitting of NMP data. However, it may be used as an "emergency " method if the backscattering system (or similar) has not been available during analysis.

Energy windows, having limits well defined from computer fitting and with the background subtracted, can be used to achieve quantitative elemental maps provided that the corresponding X-ray peaks are clearly distinguishable from the background and that few interferences occur. Even when interferences occur, the influence can, to a certain degree, be removed. Taking these reservations into account, it is possible to achieve quantitative elemental maps using only a fraction of the computer time compared to an overall X-ray data fit.

Acknowledgements.

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References

Campbell JL, Maenhaut W, Bombelka E, Clayton E, Malmqvist K, Maxwell JA, Pallon J, VandenHaute J (1986) An intercomparison of spectral data processing techniques in PIXE. Nucl. Instr. and Meth. in Phys. Res. B14 (1986) 204-220.

Chen E (1993) Inhibition of enzymes by short-wave optical radiation and its effect on the retina. Doctoral Thesis, St Erik's Eye Hospital and the Department of Medical Biophysics, Karolinska Institute, Stockholm, Sweden.

Grime GW, Watt F, Wogelius RA, Jamtveit B (1993) Processing microPIXE linescan data - studies of arsenic zoning in skarn garnets. Nucl. Instr. and Meth. B77 (1993) 410-414.

Johansson GI (1982) Modification of the HEX-program for fast automatic resolution of PIXE-spectra. X-ray Spectrum 11 (1982) 194-200.

Lindh U (Editor) (1993) Proceedings of the Third International Conference on nuclear microprobe technology and applications, June 1992, Uppsala, Sweden. Nucl. Instr. and Meth. B77 (1993)1-556.

Lövestam NEG (1989) Development of the Lund Proton Microprobe Scanning and Data Acquisition System. Nucl. Instr. and Meth. B 36 (1989) 455-470.

Lövestam NEG, Swietlicki E (1992) Off-line data evaluation of elemental maps obtained from scanning nuclear microprobe analyses. Scanning Microscopy Int. Vol. 6 (1992) 607-624.

Malmqvist KG, Hyltén G, Håkansson K, Knox JM, Larsson NP-O, Nilsson C, Pallon J, Schofield R, Swietlicki E, Tapper UAS, Yang C-Y (1993) Dedicated accelerator and microprobe line. Nucl. Instr. and Meth. B77 (1993) 3-7.

Pallon J (1990) A scanning proton microprobe data sorting and display system. Nucl. Instr. and Meth. B44 (1990) 377-391.

Ryan CG, Jamieson DN (1993) Dynamic analysis: on-line quantitative PIXE microanalysis and its use in overlap-resolved elemental mapping. Nucl. Instr. and Meth. B77 (1993) 203-214.

Themner K, Malmqvist KG (1986) Determination of local areal densities of heterogeneous biological samples by elastically backscattered protons in PIXE microanalysis. Nucl. Instr. and Meth. B15 (1986) 404-406.

Van Espen PJM, Janssens KHA (1993) Spectrum evaluation. In: Handbook of X-Ray Spectrometry. Methods and Techniques, ed. Van Grieken, RE and Markowicz AA, Marcel Dekker Inc., New York, 181-293.

Discussion With Reviewers

C. Ryan: Would you set energy windows for each element manually for each sample analyzed, or would you use a

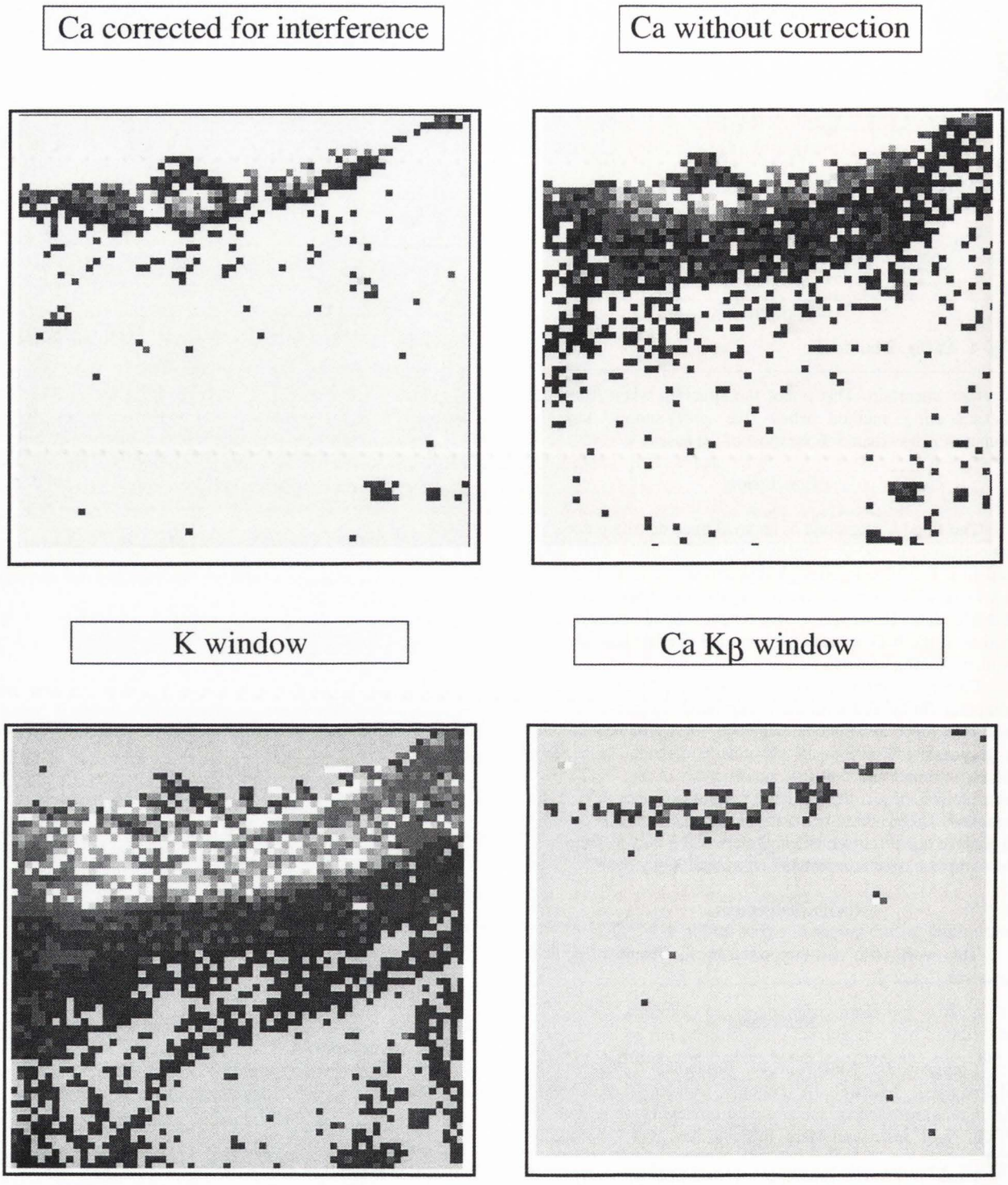

Fig. 6. Elemental map showing the Ca distribution corrected for K interference. For comparison, Ca without correction and K are shown. One can note the strong influence of K on the uncorrected Ca map. Also compare the corrected Ca map with the Ca K_β window, which is free of interference, but has poor statistics.

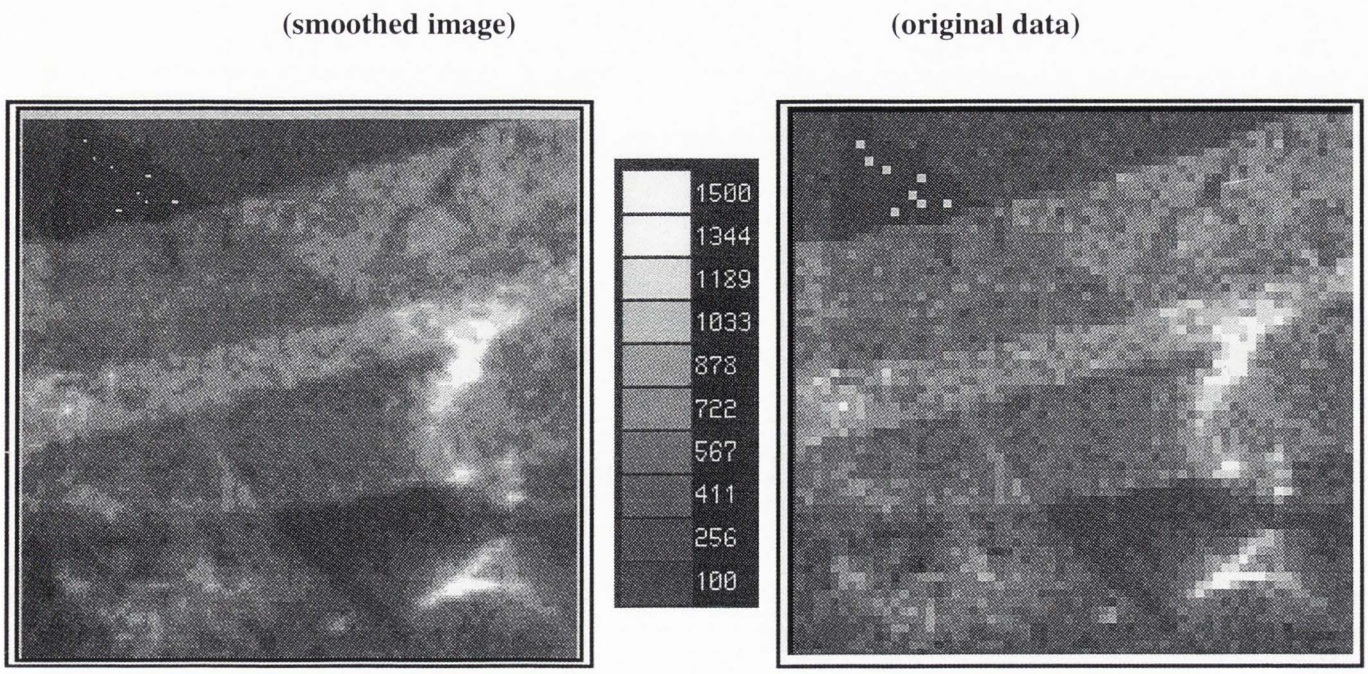

Fig. 7. Elemental map of retina 1 sample showing its areal mass density as measured by proton backscattering. The retina is the broad, light structure going horizontally in the image having a still lighter narrow band in the middle. Note the difference in backing thickness, appearing as a dark band going on the diagonal, being caused by a rupture in the foil.

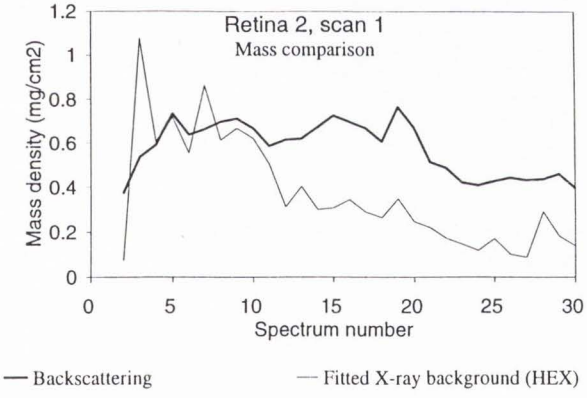

Fig. 8. Comparison of the mass density as measured by backscattering and by using the fitted PIXE background.

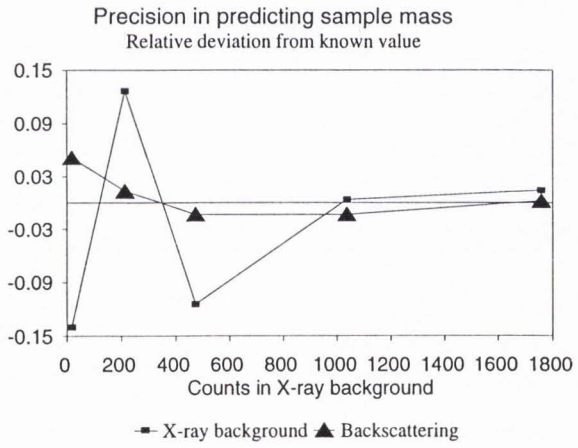

Fig. 9. Relative precision in predicting known sample mass densities as a function of the number of background counts in the X-ray spectrum. As a comparison, the precision using backscattered protons is also shown.

predefined library of windows? How are the background sample points (or energy windows) determined, or are they set for each sample following a subjective assessment by the analyst?

Authors: The first step is to do a fitting of the full scan spectrum using the code HEX. This code automatically fits both Gaussian peaks, their energy width (FWHM), background and energy calibration to the given spectrum, using a physical model. Secondly, the energy windows to be used for sorting into elemental maps are set according to the HEX fit for the elements that are of interest. The background will then automatically be those channels that limits the energy window. Assuming that all samples are analyzed at the same time, the energy calibration will not change. So in short, the window (and background) definitions are taken from a good fit of a spectrum having good statistics.

C. Ryan: Is the overlap correction done manually, on a case by case basis, by the analyst? Have you considered using the Influence Coefficients approach used in XRF, or the Dynamic Analysis method by Ryan and Jamieson (1993) to do more systematic correction for elemental overlaps?
Authors: Yes, the overlap corrections done in this work are made "manually"; the analyst has to specify for the sort/display code to show an image that is calculated from the elemental maps of K K_α and Ca K_α, and how to calculate, however, the basic operations used (intensity scaling of maps, subtraction, etc.) were already available in the code. We have considered using the Dynamic Analysis, but that work is awaiting completion of a software installation.

U. Lindh: How large is the error introduced by estimating the background by averaging the contents of the channels limiting the energy window?
Authors: This is a rather complicated question to answer, because it depends on what you are comparing with. If you have a spectrum and use a linear background under a peak instead of an empirical or non-linear you can find a number (error) related to the peak height and the background height. In the case of elemental maps you normally do not use any background at all, or a simple model like here. So we actually mean that we reduce the error instead of introducing it by applying a background subtraction, even if a simple model is used.

U. Lindh: When you removed the K K_β interference from the Ca K_α peak, was it possible to use the Ca K_β to make another comparison?
Authors: Yes, it is possible in principle, and can be seen in figure 6 by comparing the Ca K_α elemental map with the corresponding Ca K_β map. However, due to the few counts/pixel in the Ca K_β map, it may be a numerically very dangerous task to use this data to enhance the Ca K_α elemental map, although it is possible.

U. Lindh: Can you give an estimate of the variation in the scaling factor approaching trace concentrations?
Authors: This question relates to the problem of estimating the peak and background area and the question of (too) few pulses accumulated in a pixel, discussed above. The uncertainties from those two sources are added and influence the final, calculated concentration value, but strictly speaking, the scaling factors do not depend on the concentration at all.

G. Roomans: The authors mention that there are more problems with Cl than with K. Is this due to the more non-linear shape of the continuum under the Cl peak, or with the inability to completely resolve the S and Cl peak or the Cl K_α and Cl K_β peak, or with lower concentrations of Cl compared to K?
Authors: It is not likely to be due to concentration differences, and as discussed already, knowledge of the background and the peak area is critical for a precise quantification. Thus both the more non-linear background shape and the peak shape for Cl may contribute to the variation in the scaling factor.

U. Lindh: I agree with you that the tailing is an important problem in spectrum evaluation of small peaks with large neighbours. You say that increasing the number of point spectra may be a solution. Are there, according to your experience, situations where this approach fails?
Authors: This again relates back to the question of finding the "true" background. The important thing when selecting point spectra is to get examples of as many different concentrations as possible, so that an estimation of the mean and standard deviation of the scaling factors may be calculated. However, if the estimated standard deviation is large, it indicates that it is not meaningful to produce a quantitative elemental map using the method described in this paper.

G. Roomans: How does the fitting method used in PIXE compare with that commercially available for the electron microprobe? Can electron microprobe peak fitting methods be used for PIXE spectra?
Authors: The deconvolution codes used for PIXE spectra are normally more complex than similar codes for electron induced X-ray spectra, because of the differences in the physics giving rise to the X-ray spectra (e.g. the differences in electron Bremsstrahlung background), thus the latter ones has not been used for evaluating PIXE spectra.

G. Roomans: How much time (in per cent) does the authors new method save?
Authors: Using this method we have to compute the results from 50 - 150 spectra instead of 4096. If we neglect the time necessary to calculate the calibration factors from the fitted data (which is short compared to the other operations), the time saved will be 96 - 99 %.

U. Lindh: Why are you putting the sample behind two supporting foils? Is it because the sample will not stick to one foil? Are there any drawbacks?
Authors: Yes, the retina samples are rather large, and may change their shape during irradiation. The sandwich technique prevents us from loosing the sample. The main drawbacks are a partly increased background, doubled risk of contributions from the foil, and eventually a partly increased sample temperature.